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Biochimica et Biophysica Acta 1284 (1996) 29–34

BBA Biochimica et Biophysica Acta

Europium chelate-loaded liposomes: a tool for the study of binding and integrity of liposomes

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Received 15 February 1996; revised 13 May 1996; accepted 15 May 1996

Abstract

Using the biotin-streptavidin interaction as a model, we investigated the suitability of lanthanide chelates as encapsulated liposomal labels in liposome-based binding assays. Large unilamellar phospholipid:cholesterol liposomes containing europium-DTPA chelate and biotinylated phosphatidylethanolamine were prepared by detergent dialysis. The resulting Eu-liposomes (∅120 nm) bound specifically to streptavidin in microtiter wells as measured by time-resolved fluorometric assay (TRF). The intensity of fluorescence released from the bound liposomes was dependent on the concentration of biotin in the liposome membrane, the concentration of europium entrapped in the liposomes, the incubation time and the amount of liposomes used in the assay. The sensitivity of the TRF assay allowed the detection of binding of attomole quantities of liposomes. The streptavidin-immobilised liposomes subjected to porcine pancreatic phospholipase A_2 (EC 3.1.1.4) and detergents displayed a dose-dependent release of the encapsulated europium. Lanthanide-chelate-liposomes should prove useful for studies addressing binding and stability of liposomes.

Keywords: Liposome; Streptavidin; Biotin; Europium-chelate; Lanthanide; Time-resolved fluorometry; Detergent; Phospholipase A_2

1. Introduction

Liposomes can incorporate fluorescent labels and other indicator molecules both as encapsulated within the aqueous space (polar compounds) or as embedded in the lipid membrane (lipophilic species) [1]. The ease of preparation and the possibility to introduce molecular recognition elements such as haptens, glycans, peptides and membrane proteins on the surface of the liposomes has spurred an interest in using liposomes in biotechnological applications [2–5]. Besides applications involving drug delivery (for review see [6,7]), and more recently, gene therapy [8], the potential for signal amplification inherent to the liposomal encapsulation of different indicator molecules has also prompted attemps to develop immunological and other assays based on targeted binding of label-carrying liposomes [9–11].

Fluorescent lanthanide chelates have a long-lived fluorescence with a large Stokes' shift, facilitating the measurement of the fluorescence in a time-resolved mode resulting in minimal interference by background fluorescence in biological samples and by the scatter of excitation light [12]. The stability of lanthanide chelates contrasts that of commonly used organic fluorophores such as fluorescein and rhodamine and leads to increased shelf-life of the labelled species. Together, these properties have led to increased use of fluorescent lanthanide chelates in research and diagnostics [13]. For a lanthanide chelate-based assay, the biomolecule is labelled with a nonfluorescent lanthanide chelate by using conventional coupling chemistries. After appropriate binding and washing steps, the lanthanide ion is released from the labelled species to form a highly fluorescent chelate in a micellar environment [14].

In the present study, we describe the preparation and properties of europium-chelate-loaded liposomes which can be used to amplify the fluorescent signal in binding assays. Furthermore, by exploiting the strong interaction between biotin and streptavidin, we describe the use of Eu-liposomes as a platform to develop sensitive solid-phase assays based on liposome lysis.

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PII S0005-2736(96)00104-6

2. Materials and methods

2.1. Materials

Egg yolk L-phosphatidylcholine (PC) and L-phosphatidylethanolamine (PE) were purchased from Avanti Polar Lipids (Birmingham, AL, USA). *N*-(Biotinoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine triethylammonium salt (B-PE) was from Molecular Probes (Eugene, OR, USA). Cholesterol (CHO) was from Sigma. L-α-Dipalmitoyl-[2-palmitoyl-1-^{14}C]phosphatidylcholine ([^{14}C]PC) with a specific activity of 2.054 Gbq/mol was from New England Nuclear-DuPont. Europium (III) chloride hexahydrate ($EuCl_3$) was supplied by Aldrich and diethylenetriamine pentaacetic acid (DTPA) by Fluka. Porcine pancreatic phospholipase A_2 (PLA_2, EC 3.1.1.4) was purchased from Sigma as an ammonium sulfate suspension and was used after extensive dialysis.

DELFIA® streptavidin-coated microtitration strips (8x12 wells) and DELFIA® enhancement solution [15 μM 2-naphthoyltrifluoroacetone (2-NTA), 50 μM tri-*n*-octylphineoxide (TOPO) and 0.1% (v/v) Triton X-100 in 0.1 M acetate-phthalate buffer, pH 3.2] were obtained from Wallac (Turku, Finland).

Triton X-100 and *n*-octyl β-D-glucopyranoside (OG) were supplied by Boehringer-Mannheim and Sigma, respectively. Polystyrene microstrips were purchased from Labsystems (Helsinki, Finland).

2.2. Preparation of Eu-liposomes

Liposomes were prepared by detergent dialysis as follows. First, a lipid film PC/PE/CHO (molar ratio, 17.3:2.7:8) and the appropriate amount of B-PE (0–5 mol% of total lipid) was dried from a chloroform solution on the walls of glass container by rotary evaporation. The lipid film was dispersed in 0.15 M NaCl, 20 mM Hepes pH 7.4 (buffer A) (1 mg/ml) and solubilized in 1% (w/v) of OG containing the appropriate concentration of Eu-DTPA chelate (molar ratio 1:1; 1, 10 and 50 mM) in buffer A. The dialysis was performed in a LIPOSOMAT dialyser (Dianorm, Munich, Germany) equipped with a cellulose membrane (cut-off 10 kDa). To enable a strict control over the concentration of encapsulated europium chelate, the Eu-DTPA concentration was maintained at an equal concentration on both sides of the membrane during the first 60 min of the dialysis. During this time, the liposomes formed as the suspension became slightly opalescent. Subsequently, non-encapsulated europium chelate was removed by dialysis against buffer A, containing equal concentration of DTPA, until no more free europium chelate was observed in the dialysate as measured by TRF (about 3 h). The resulting suspension of Eu-DTPA-loaded liposomes (Eu-liposomes) were collected by ultracentrifugation (150 000 × *g*, 2 h, 4°C), suspended in buffer A and re-centrifuged, and finally suspended into 500 μl of buffer A. Results corresponds of at least three separate liposome batches.

The recovery of liposomes in the ultracentrifugation was determined by using [^{14}C]PC as a tracer and by Stewart's assay [15] to measure the lipid concentration. No losses were observed during this process.

2.3. Time-resolved fluorometric assay

The amount of europium in the samples was measured by using a time-resolved fluorometric DELFIA assay [16]. Briefly, 190 μl of DELFIA enhancement solution was added to 10 μl of the sample. In the detergent-containing enhancement solution the europium is released as highly fluorescent micellar complex [17]. After 5 min of shaking at room temperature, samples were measured with an ARCUS 1230 time-resolved fluorometer (Wallac, Finland). In this instrument, the sample is pulsed 1000 per s with an excitation light of 340 nm. In the period between flashes, 613-nm fluorescence of the sample is measured for 400 μs after a delay time of 400 μs that allows the short-lived background fluorescence to decay. The photons counted during one second are recorded and expressed as counts per second (cps) [16].

2.4. Liposome binding assay

The binding of Eu-liposomes to streptavidin was measured by using a time-resolved fluorometric assay as outlined in Fig. 1. Briefly, 100 μl of Eu-liposomes (0.6 μg of phospholipid /well) were incubated in streptavidin-coated microtiter wells for 2 h at room temperature under gentle shaking. The wells were washed three times with PBS (0.9% NaCl, 50 mM phosphate buffer, pH 7.4) using an automatic plate washer (1296-024, Wallac, Finland). Then, 200 μl of DELFIA enhancement solution and after 5 min of shaking at room temperature, samples were measured for fluorescence as above. For control experiments, microtiter wells previously coated overnight at 4°C with 100 μl of BSA (42 μg/ml) in 0.1 M Na-bicarbonate buffer, pH 9.6 were used in instead of streptavidin coated wells. Unless otherwise indicated, all experiments were carried out with 0.6 μg of phospholipid /well.

2.5. Liposome permeability measurement

Eu-liposomes (1 mol% of B-PE, 10 mM Eu-DTPA) were bound to streptavidin coated wells as described above. Then, 200 μl of buffer A containing different concentrations of Triton X-100 or OG was added. After a 1-h incubation at room temperature, the wells were washed three times with PBS and the europium was released by adding 200 μl of DELFIA® enhancement solution and the fluorescence signal was measured. Also, the effect of PLA_2 was tested in a similar manner by adding 100 μl of buffer A containing 1 mM $CaCl_2$ and different amounts of

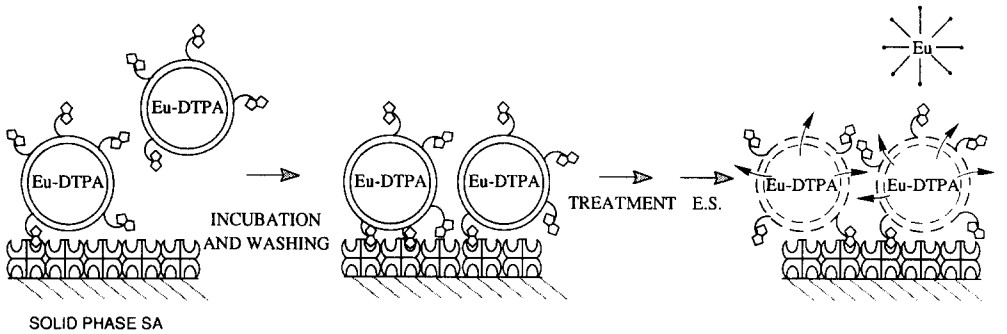

Fig. 1. Schematic representation of liposomal assay protocol. Liposomes (PC/PE/CHO) including variable amount of B-PE (0–5 mol% of the total lipid) and Eu-DTPA chelate (1–50 mM) were incubated (100 μl/well) in streptavidin coated microtiter wells. After a 2-h incubation at room temperature, the unbound liposomes were removed by washing. The fluorescence of the immobilised Eu-liposomes was developed by the addition of DELFIA® enhancement solution (E.S.), and the fluorescence was measured in an ARCUS time-resolved fluorometer. The treatment refers to the addition of detergents and PLA_2, as described in the text (see Section 2).

PLA_2 and incubated for 3 h at 37°C under shaking. Thereafter, the assay was done as above.

3. Results

3.1. Preparation of Eu-liposomes

Eu-liposomes were prepared by dialysis from an OG solution of lipids mixture and Eu-DTPA chelate (1–50 mM). The detergent was removed during dialysis in the Liposomat resulting in the formation of unilamellar liposomes containing the entrapped marker in a reproducible manner. Eu-liposomes negatively stained with 1% potassium phosphotungstate (pH 7.4), appeared as a relatively homogeneous population of apparently unilamellar liposomes (120 ± 30 nm, $n = 400$) when studied by transmission electron microscopy (Fig. 2).

3.2. Characterisation of the Eu-liposomes

To demonstrate the usefulness of lanthanide-chelate-loaded liposomes in liposomal binding studies, streptavidin-biotin interaction was used as a model system (Fig. 1). Biotinylated phosphatidylethanolamine was incorporated (0–5 mol% of the total lipids) into the liposomes during their preparation. The binding of Eu-liposomes to streptavidin was then measured as the amount of the europium released from the bound liposomes after incubating biotinylated liposomes in streptavidin coated wells. The release was achieved by addition of DELFIA dissociative enhancement solution containing detergent. Eu-liposomes containing 1 mol% of biotinylated PE bound to streptavidin but not to BSA whereas liposomes containing no biotinylated PE did not bind to streptavidin nor to BSA (Fig. 3A). Thus, both biotin and streptavidin were required for the binding of liposomes. Furthermore, the presence of a molar excess of free biotin and biotinylated alkaline phosphatase inhibited the binding of Eu-liposomes to immobilised streptavidin (data not shown).

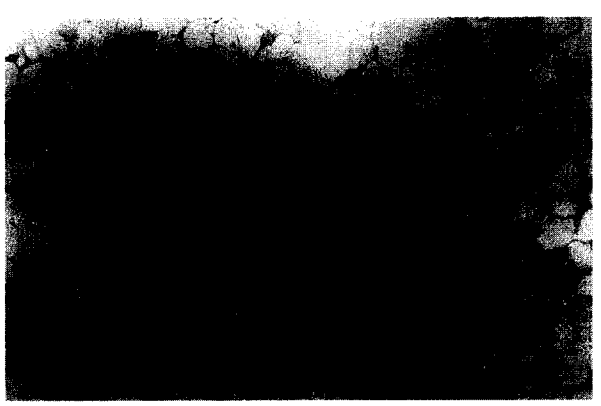

Fig. 2. Electron micrograph of negative-stained Eu-liposomes. Liposomes containing 1 mol% of B-PE and 10 mM Eu-DTPA were prepared by detergent dialysis. The bar corresponds to 200 nm.

The binding of Eu-liposomes (1 mM Eu-DTPA, 60 μg phospholipid per well) to streptavidin increased with increasing content of biotinylated PE up to 1 mol% of B-PE (Fig. 3B). As expected, the fluorescence signal obtained from bound Eu-liposomes (1 mol% B-PE) was also dependent on the concentration of entrapped Eu-DTPA at the concentration range of 1–50 mM (Fig. 3C).

The dose-dependency of the binding of Eu-liposomes (10 mM Eu-DTPA) to streptavidin is shown in Fig. 4A. Increasing the amount of Eu-liposomes resulted in increased fluorescence signals and reached a maximal level at 12 μg phospholipid/well. The fluorescence obtained from the bound Eu-liposomes was correlated to actual europium concentrations by using a standard curve (Fig. 4A inset). We assumed the concentration of europium in the liposomes equal to the initial concentration of the label because during the formation of the liposomes, the label is present at equal concentration on both sides of the dialysis membrane. Therefore, from the size (∅ 120 nm) and the loaded concentration of europium (10 mM), one liposome is calculated to contain approximately 3000 Eu^{3+} ions. Considering binding signals exceeding the background by

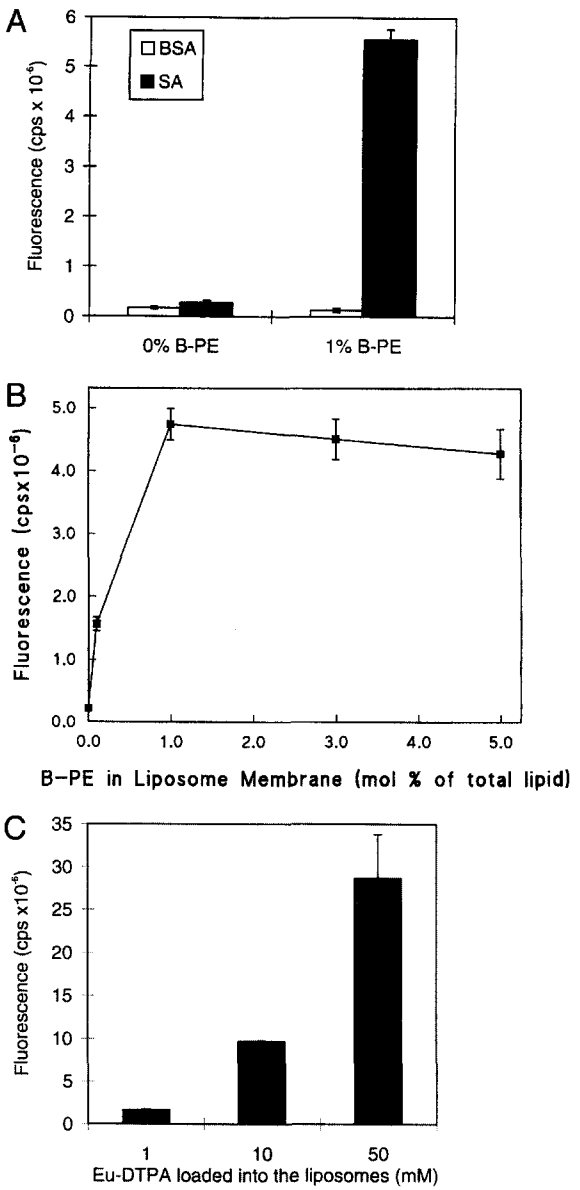

Fig. 3. Characterisation of the binding of Eu-liposomes to streptavidin. All values are means of three measurements (±S.D.). (A) Specificity of the binding. Eu-liposomes (1 mol% B-PE) and control liposomes were loaded with 1 mM Eu-DTPA and bound to BSA and streptavidin coated wells. (B) Dependency of the binding of liposomes on the biotin-content. Eu-liposomes containing different amounts of B-PE (0–5 mol%) bound to streptavidin. (C) Different concentrations of entrapped Eu-DTPA. Eu-liposomes (6 μg phospholipid/well; 1 mol% B-PE) loaded with 1, 10 and 50 mM of Eu-DTPA were bound to streptavidin.

a factor of three as significant, the binding of four attomoles of liposomes was easily detected. The background fluorescence in our assay is defined as the binding of non-biotinylated liposomes to streptavidin or by binding of biotinylated liposomes to BSA and is about 5000 cps. Next, the kinetics of the binding of Eu-liposomes to streptavidin was studied. Using 6 μg of phospholipid /well of Eu-liposomes (1 mM Eu-DTPA), the binding reached its maximal level within a 2-h incubation at room temperature (Fig. 4B).

After a 2-month storage of the Eu-liposomes at 4°C the fluorescence signal obtained by the binding assay was still about 90% of the value obtained with fresh Eu-liposomes as measured by TRF. Eu-liposomes containing different amounts of B-PE showed similar stability indicating that the presence of B-PE did not affect to the stability of the liposomes.

3.3. Liposomal permeability measurements

The potential use of the streptavidin-bound Eu-liposomes for monitoring membrane permeability was studied by subjecting immobilised liposomes to membrane-destabilising agents. The effects of the treatments were measured as the europium fluorescence signal remaining associated with liposomes. First, the effects of two nonionic

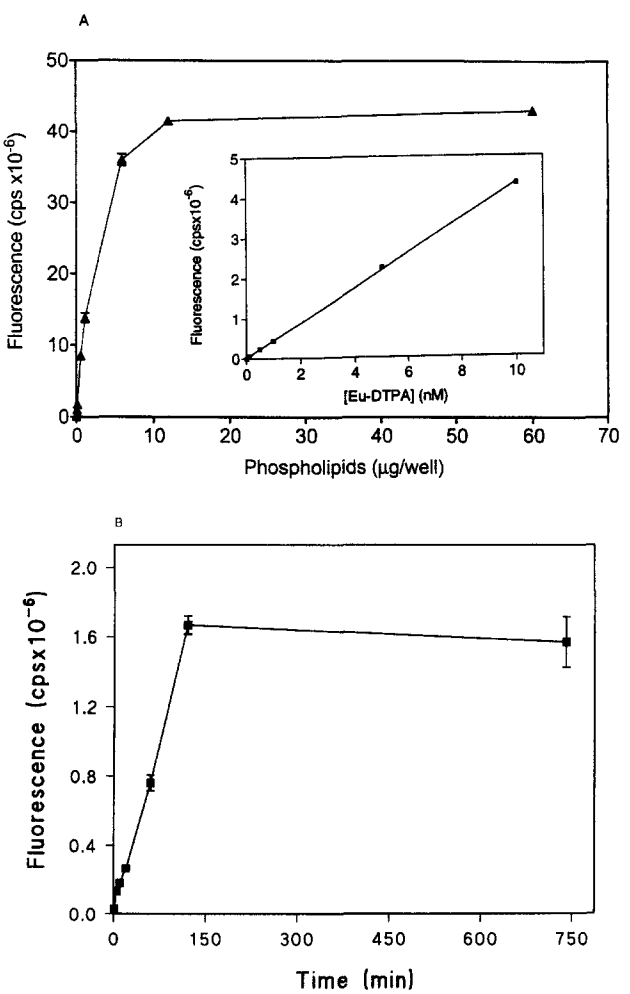

Fig. 4. Characteristics of the liposome binding assay. (A) Dose-response curve of Eu-liposomes loaded with 10 mM of Eu-DTPA to streptavidin. Inset shows the standard curve of Eu-DTPA. (B) Time-course of the binding Eu-liposomes (60 μg phospholipid/well) containing 1 mol% B-PE and 1 mM of Eu-DTPA. Inset corresponds to a standard curve of Eu-DTPA.

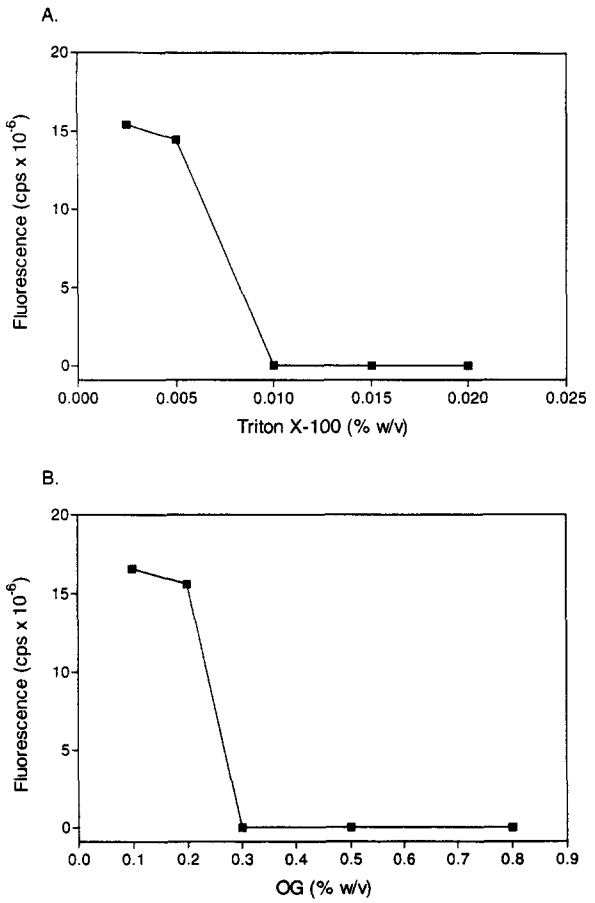

Fig. 5. Effect of detergents on the release of europium from immobilised liposomes. Streptavidin-bound Eu-liposomes containing 1 mol% B-PE and 10 mM Eu-DTPA were subjected to (A) Triton X-100 and (B) OG (200 μl/well), and the bound fluorescence was measured as in Fig. 1.

detergents, Triton X-100 and OG were studied. The critical micelle concentrations (CMC) of the detergents are 0.013% (w/v) and 0.42% (w/v), respectively [18]. For both detergents, a small decrease in the fluorescence was observed at concentrations below the respective CMCs, whereas at concentrations above the CMC, a total release of europium occurred (Fig. 5A,B).

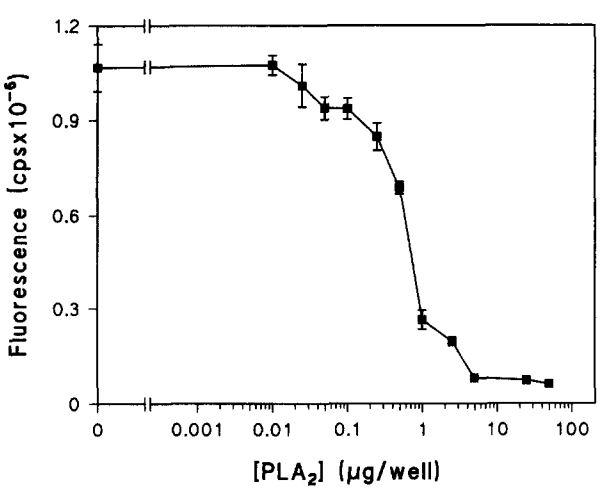

Fig. 6. Effect of the PLA_2 on release of europium from immobilised Eu-liposomes. Different amounts of PLA_2 (100 μl/well) were incubated with immobilised Eu-liposomes containing 1 mol% B-PE and 10 mM Eu-DTPA, and the bound fluorescence was measured as in Fig. 1.

Table 1
Effect of PLA_2 on the liposomal permeability

$[CaCl_2]$ (mM)	Fluorescence (cps × 10^{-6})	Reduction (%)
Buffer [a]	1.5	–
0	1.2	20.9
1	0.2	86.1
2.5	0.2	87.2
5	0.2	88.9
10	0.2	85.8

Immobilized Eu-liposomes containing 1 mol% B-PE and 10 mM Eu-DTPA were subjected to 2.4 μg/well PLA_2 solutions in the presence of different amounts of Ca^{2+} (0–10 mM) (see Section 2).
[a] Blank corresponds to buffer without enzyme.

Next, we studied the effect of phospholipase A_2 (PLA_2) which catalyses the hydrolysis of the fatty acid ester in position 2 of 3-*sn*-phospholipids and thereby increases membrane permeability [19]. A dose-dependent release of europium was observed on PLA_2 addition starting at a dose of 50 ng of enzyme per well and a total release of encapsulated europium occurred at an enzyme concentration of 2.4 μg/well (Fig. 6). In accordance with the strict Ca^{2+}-dependency of PLA_2 [20], no release of europium was observed from Eu-liposomes treated with PLA_2 (2.4 μg/well) in the absence of Ca^{2+} (Table 1).

4. Discussion

In the present work, we have studied the use of europium as an encapsulated label as a step towards the development of sensitive liposome-bound assays. With this aim, we prepared lanthanide-chelate-loaded liposomes doped with biotin and characterised their binding to polystyrene-bound streptavidin. Our preliminary experiments, in agreement with a previous short report [21], indicated that europium has to be chelated in order to obtain stable liposomal encapsulation. This is probably due to the interaction between the phospholipid headgroup and free europium ion interfering with the formation of the liposomes [22]. The use of DTPA as chelating compound proved compatible with the preparation of liposomes and provided stable encapsulation of europium. We chose detergent dialysis as the method for the preparation of liposomes because it allows a good control over the formation of liposomes which are unilamellar and relatively homogeneous in size. Importantly, by detergent dialysis it is also easy to incorporate functional membrane proteins in the lipid bilayer. Generally, wasteful use of the label to be encapsulated is a drawback of the dialysis method. In our case, however, this is not a major problem because europium chelates are relatively inexpensive.

The fluorescence signal obtained in the TRF-assay was dependent on the amount of liposomes and encapsulated europium as well as the concentration of biotin on the surface of the liposomes. When liposomes were loaded with 10 mM europium chelate specific binding of attomole amounts of liposomes was detected highlighting the sensitivity obtained by combining time-resolved fluorometry with signal amplification by liposomal encapsulation of europium. Conventionally, europium-chelate-labelled proteins contain approximately 1–10 labels/molecule, whereas a 100-nm liposome with the entrapped 10 mM Eu-DTPA contains 10^3 label molecules. The europium content can be varied in the range of 10^2–10^5 europium molecules per liposome either by varying the initial concentration of europium in the solution and the size of the liposomes.

The potential usefulness of Eu-liposomes immobilised via biotin-streptavidin interaction to microtiter wells as a format for liposomal release assays was demonstrated by using detergents and phospholipase A_2 to induce membrane permeability. Liposomes encapsulating marker molecules and decorated with antigen or antibody are used as analytical reagents to amplify the binding signal in liposomal immunoassays (LIA) [23,24]. Specifically, in liposomal immunolysis assay (LILA) [25], the encapsulated marker is released by the lytic activity of serum complement induced by the formation of immunocomplex. In these liposome assays, a variety of encapsulated labels has been used as quantitative indicators (for review see Ref. [26]). In general, conventional fluorescent labels such as carboxyfluorescein, sulforhodamine and calcein suffer from limited detection range (micromolar to nanomolar range) because of high background fluorescence and self-quenching [27]. On the other hand, this self-quenching can be exploited in studies of liposomal permeability and in liposomal immunoassays [28]. In the present study, we have demonstrated that lanthanide chelates when used as entrapped liposomal labels provide several advantages, most remarkably, inexpensiveness, stability and extremely sensitive measurement by using time-resolved fluorescence. Recently, we have also showed that Eu-immunoliposomes can be used as specific reagents in an immunoassay [29].

In conclusion, by combining the high sensitivity of time-resolved fluorescence measurement of lanthanide chelates with encapsulation of large number of label molecules, the Eu-liposomes provide a convenient tool to studies on liposome binding and membrane permeability.

Acknowledgements

We thank Prof. Hans Söderlund for discussions and comments on the manuscript. We thank the Department of Electron Microscopy, University of Helsinki, for providing EM facilities. A.O. was supported by a grant from CIRIT, Autonomous Government of Catalonia, Spain.

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